1. Introduction

The immune system is able to constantly face a wide variety of infections. Moreover, diverse pathogens pose different challenges for which the immune system needs to elicit targeted responses. One of the mechanisms for tailoring the immune response to different threats is cytokine induced cell polarisation (1). This process is mediated by proteins which act as messengers between immune cells, and is key for the response of cells to environmental cues. Moreover, cytokine induced polarisation is also involved in driving pathological responses to self-antigens that lead to autoimmunity (2).

In this chapter I summarise our current understanding of cytokine induced cell polarisation, focusing on two cell types which have been associated to autoimmune disease: $CD4^+$ T cells and macrophages, (3, 4). Next, I describe the role of cytokine induced polarisation in autoimmunity. Finally, I state the aims and experimental design of the present study.

1.1 Cytokine induced polarisation of $CD4^+$ T cells

1.1.1 $CD4^+$ T cells are key players of the adaptive immune response

T cells are a subtype of lymphocyte that coordinates multiple aspects of the immune response (1). The stimuli that trigger T cell responses occur in secondary lymphoid organs, where professional antigen presenting cells (APC) such as macrophages and dendritic cells (DCs) present peptides to T cells (5). For successful activation T cells need to receive two signals from APCs. The first signal is triggered when T cell receptor (TCR) recognizes antigen presented in the context of major histocompatibility complex (MHC) molecules. In addition, APCs provide a second signal known as costimulation via interaction of the receptor CD28 on the surface of T cells with CD80 and CD86 molecules expressed on the surface of APCs (6-8). Following stimulation, T cells undergo clonal expansion and acquire specialized functions (1).

$CD4^+$ T cells are sometimes referred to as T “helper” (Th) cells due to their role in recruiting and modifying the function of other cell types (1, 9). For example, $CD4^+$ T cells can instruct class switching of B cells, specifying which type of antibody should be secreted (10). Moreover, $CD4^+$ T cells can also provide “help” to macrophages by promoting phagocytosis (11). These aspects of $CD4^+$ T cell function are mediated by cytokines, proteins which bind

to receptors in the surface of target cells and modify gene expression via transcription factors (TFs) such as signal transducers and activators of transcription (STATs) (12).

1.1.2 Cytokines guide the differentiation of $CD4^+$ T cells

Multiple subsets of $CD4^+$ T cells are generated in response to infection, each of them with different cytokine secretion profiles and in charge of providing a specific type of "help" (13). The specialization of $CD4^+$ T cells into subsets is itself guided by cytokines present in the microenvironment during and after T cell activation and is referred to as cytokine induced T cell polarisation (13). Polarisation of $CD4^+$ T cells to different subsets with specialised functions was first described in 1986 by Mosmann et al. who reported the existence of two types of $CD4^+$ T cells with characteristic cytokine secretion profiles (14). These cells were named Th1 and Th2, and were defined by their secretion of IFN-γ but not IL-4, and IL-4 but not IFN-γ, respectively (15, 16). Several other studies expanded upon these observations to include IL-10, IL-5, and IL-13, which are also secreted by Th2 cells (17). Th1 cells were found to also secrete IL-2 and GM-CSF (14). Despite their initial description in cell lines, Th1 and Th2 cells were later identified *in vivo* in mouse models of infection (18, 19) and equivalent cell populations have been described in humans (20, 21). *In vitro*, these cell types can be induced by $CD4^+$ T cell activation in the presence of IL-12 or IL-4, respectively (22-24). This paradigm has been extended to include Th17 cells, characterised by secretion of IL-17 but not IL-4 or IFN-γ, and which arise as an independent lineage upon $CD4^+$ T cell activation in the presence of TGF-β, IL-1β and IL-23 (25-27). Furthermore, induced regulatory T (iTreg) cells develop upon T cell activation in the presence of TGF-β (28). More recently, Th9 and Th22 cells have also been described as subsets that secrete IL-9 and IL-22 respectively, though their phenotypic stability is still debated (29, 30). The total number of T cell subsets thus remains unclear and continues to be an area of debate.

$CD4^+$ T cell polarisation is also affected by the intensity of TCR signalling (31). This was first suggested by *in vitro* stimulation of mouse $CD4^+$ T cells, which showed that polarisation efficiency is proportional to stimulation time (32). Further experiments in mice showed that strong TCR stimulation, as measured by Ca^{2+} flux, generally enhances Th1 polarisation, while Th2 polarisation is favoured by weak stimulation (33). However, it remains unclear whether other lineages could also fit in this model. A study of human $CD4^+$ T cell stimulation with different strengths of TCR signalling suggested that Th17 differentiation is favoured by low intensity stimulation (34). It has even been proposed that signalling strength might be

more important than the cytokine milieu in determining T cell fate, since some degree of differentiation is achieved even upon cytokine blockade (33). Nonetheless, this hypothesis needs further validation.

The current paradigm proposes that polarisation of $CD4^+$ T cells is a differentiation processes which generates stable cell lineages (13). T cell polarisation is triggered by the expression of specific TFs called “master regulators”, which are able to modify the chromatin landscape of activated T cells (13). T-bet and GATA-3 regulate Th1 and Th2 differentiation, respectively (35, 36), while Th17 differentiation is controlled by RORγ (37). PU.1 and the aryl hydrocarbon receptor (AHR) have been proposed as master regulators of the Th9 and Th22 lineages respectively (30, 38). Moreover, TGF- β induces iTreg differentiation, most likely mediated by upregulation of the TF FoxP3 (39, 40). However, recent evidence from epigenetic studies suggests that to carry out their function these master regulators need other TFs such as STATs, which modify the chromatin landscape and create active enhancers (41, 42). This suggests that T cell polarisation is coordinated by a complex network of TFs rather than a single master regulator. Moreover, direct interaction of TFs is also involved in fate determination. For example Th1 cells express GATA-3, but here the TF is unable to bind its target genes because of direct interaction with T-bet (43). Consequently, chromatin remodelling and TF networks play a major role in determining $CD4^+$ T cell fate.

1.1.3 DCs polarise $CD4^+$ T cells to different phenotypes in response to different pathogens

T cell polarisation states have a functional role in the immune response to infection, with each subset responding more efficiently against a specific type of pathogen (44). Th1 cells, for example, arise in response to viruses and intracellular bacteria and are able to enhance the phagocytic capacity of macrophages via secretion of IFN-γ (11). Conversely, Th2 cells enhance the mobilization of eosinophils and generate alternatively activated macrophages which clear helminths and other multicellular organisms (45). Th17 cells, on the other hand, interact with neutrophils in the immune response to extracellular bacteria via secretion of IL-17 (46, 47). Thus, T cell polarisation is a tightly controlled developmental process dependent on the pathogenic challenge. Cytokines that guide $CD4^+$ T cell differentiation are secreted by DCs, which were previously exposed to specific pathogen in the tissues and subsequently migrated to the lymph nodes (48, 49). For example, in response to stimulation with lipopolysaccharide (LPS), a component of the outer membrane of gram negative bacteria,

DCs secrete IL-12 which promotes Th1 differentiation (50). Nematode infections cause DCs to secrete high levels of IL-4, promoting Th2 responses (51).

Interestingly, it has been demonstrated that DCs secrete different cytokines throughout the immune response, changing the conditions under which T cell activation occurs (52). The early immune response is characterised by “active” DCs which secrete IL-12, while “exhausted” DCs secrete IL-4 and are characteristic of the late immune response (52). Furthermore, duration of DC-T cell interaction has been implicated in Th1/Th2 polarisation (33). *In vivo* imaging of mouse lymph nodes by two-photon microscopy has demonstrated that Th1 responses arise upon long and sustained interactions between DCs and $CD4^+$ T cells. Conversely, in Th2 responses T cells display multiple brief interactions with DCs (33). A similar study confirmed that sustained interactions cause secretion of IFN-γ by T cells (53). Together these studies suggest that T cell polarisation is guided by DCs in the lymph nodes and changes throughout the immune response.

Cytokine induced polarisation is known to modify the migratory capacity of T cells by inducing expression of specific homing receptors (54, 55). T cells can then migrate to inflamed tissues where they are exposed to different stimuli and acquire new functions. For example, a mouse model of infection showed that Th17 differentiation can be locally induced in the gut by changes in the microbiota (56). Other stimuli different from cytokines are also involved in this process. For instance, it was found that DCs in the Peyer’s patch can induce expression of the gut homing receptor CCR9 by mouse T cells (57). This phenotype was not induced by DCs from other locations. This tropism is mediated by retinoic acid produced *in situ* (58). The same is observed in the skin, where DCs process vitamin D3 into its active form $1,25(OH)_2D_3$, inducing the expression of CCR10 by T cells which can then migrate to the skin (59). This process is restricted to the skin, with $1,25(OH)_2D_3$ inhibiting T cell homing to the gut (59). Consequently, it is increasingly appreciated that upon polarisation $CD4^+$ T cells home to inflamed tissue, where differentiation is completed.

1.1.4 T cell polarisation states retain functional plasticity

T cell polarisation states are mutually exclusive. Th2 differentiation, for example, is inhibited by IFN-γ (60, 61). Moreover, Th17 differentiation requires blockade of both IFN-γ and IL-4 (25). This suggests that throughout the immune response one lineage might dominate over others. This is enhanced by the autocrine action of endogenous cytokines, which promote

self-sustained T cell polarisation via a positive feedback loop. Th1 cells, for example, secrete IFN-γ, which itself induces the expression of T-bet (62). Furthermore, IL-12 also induces the expression of its own receptor (63). Similarly, Th2 cells secrete IL-4, which enhances the expression of GATA-3 (36). Consequently, it is thought that mutually exclusive T cell phenotypes arise upon responses to different stimuli and cytokine milieus, and that each lineage is expanded by a positive feedback loop.

Despite this mutual exclusivity, T cell polarisation states are also highly plastic (3) and under certain conditions cells can acquire a new phenotype upon re-stimulation with different cytokines. For instance, GATA-3$^+$ cells committed to the Th2 lineage can still secrete IFN-γ if transferred to a mouse infected with lymphocytic choriomeningitis virus (LCMV), known to promote Th1 responses (64). This study reported stable co-expression of GATA-3 and T-bet (64). Furthermore, iTreg and Th17 cells are able to interconvert, despite their opposing functional roles (65, 66). This plasticity is likely mediated by epigenetic mechanisms (67). It has been demonstrated that CD4$^+$ T cells from one lineage suppress the secretion of cytokines from other lineages by temporarily silencing those genomic regions, as suggested by global mapping of histone marks in Th1, Th2, and Th17 mouse cells (68). However, the promoter region of master regulator TFs such as T-bet shows both accessible and repressive chromatin marks which coexist, suggesting complex regulatory mechanisms (68). In conclusion, CD4$^+$ T cell polarisation generally results in mutually exclusive lineages, but the resulting cells also retain functional plasticity and can adapt in response to their environment.

1.2 Cytokine induced polarisation of macrophages

1.2.1 Macrophages are key players of the innate immune response

The phagocyte mononuclear system is composed of monocytes and tissue macrophages, in charge of clearing infecting pathogens through phagocytosis, as well as maintaining tissue homeostasis (69). Following maturation, monocytes egress to the periphery, where they express the surface marker CD14 (70), a co-receptor for bacterial LPS (71). Monocytes have been classified into two groups: “classical” monocytes (CD14$^+$CD16$^-$), and “alternative” monocytes (CD14$^+$CD16$^+$) (72, 73). Upon tissue damage, classical monocytes are recruited to the affected area via the chemokine CCL2, where they preferentially differentiate into monocyte derived macrophages. This differentiation is mostly triggered by macrophage

colony stimulating factor (M-CSF) (72). Conversely, $CD14^+CD16^+$ monocytes seem to migrate into the tissues under healthy conditions via chemokines as CX_3CL1, and differentiate mostly into dendritic cells (DCs) (74, 75). This differentiation process is triggered by granulocyte/monocyte-colony stimulating factor (GM-CSF) and IL-4 (72).

Macrophages show phenotypic diversity across different tissues: Langerhans cells in the skin, alveolar macrophages in the lungs, microglia in the brain, and osteoclasts in the bones, among other examples (76-79). However, they share common functions. Firstly, they are professional phagocytes which participate in cellular immunity by engulfing pathogenic particles and targeting them to the phagolysosome, where they are degraded by proteolytic enzymes and reactive oxygen/nitrogen species (ROS/NOS) (80). Secondly, macrophages also mediate homeostasis by clearing apoptotic cells and debris via phagocytosis, and repairing tissue (81, 82).

In order to carry out their functions, macrophages must be activated. Activation is triggered by binding of an alarm signal such as LPS to pattern recognition receptors (PRRs), for example Toll-like receptors (TLRs) (80, 83, 84). The molecules that bind PRRs commonly belong to one of two families: pathogen associated molecular patterns (PAMPs), and damage associated molecular patterns (DAMPs) (85), with this last group consisting of intracellular antigens such as dsDNA which are released to the microenvironment upon tissue destruction. Macrophages also produce pro-inflammatory cytokines such as IL-1β and tumour necrosis factor (TNF) (86). Furthermore, their activation can be modified or fully triggered by the cytokine milieu (83, 87). This process is referred to as cytokine induced macrophage polarisation.

1.2.2 Th1/Th2 cytokines polarise macrophages to an M1/M2 phenotype

Cytokine induced macrophage polarisation was first described by Mills et al., who proposed the existence of two functional classes of macrophages: M1 and M2 (88). M1 macrophages arise upon stimulation with IFN-γ in the presence or absence of LPS, and have enhanced inflammatory and phagocytic capacity (11, 83). On the other hand, polarisation with IL-4, IL-10 or IL-13 generates M2 macrophages (83, 89), which are central to the immune response against parasites (90). M2 macrophages also mediate tumour immune evasion (91). The M1/M2 nomenclature comes from the fact that, as described before, IFN-γ is produced by Th1 cells while IL-4, IL-10 and IL-13 are signature Th2 cytokines. Thus, a functional equivalence between $CD4^+$ T cell and macrophage subsets was proposed (88).

Subsequently, the response of macrophages to IFN-γ and IL-4 has been characterised in more depth. For instance, it is now known that M1 polarisation is mostly mediated by STAT1, while STAT6 coordinates M2 polarisation (87, 92). Furthermore, the mammalian target of rapamycin complex 1 (mTORC1) is also involved in macrophage activation, with constitutive induction of mTORC1 preventing development of the M2 phenotype (93). Transcriptional profiling of M1/M2 macrophages has also been performed using microarrays, which revealed crucial differences between both phenotypes such as the expression levels of mannose receptors and cyclooxygenases (94). Efforts to identify M1 and M2 specific surface markers have suggested M2 macrophages express the mannose receptor Mrc2, as well as Ym1, FIZZ1, and Arginase 1 (Arg1) (94-96). On the other hand, the inducible nitric oxide synthase (iNOS) is the gold standard for M1 identification (83). However, Arg1 is also expressed in other subsets, such as tissue resident macrophages and in other cells involved in the responses to intracellular pathogens (97), questioning the validity of single surface markers. Thus, an in-depth description of the full transcriptome upon M1/M2 polarisation is still necessary.

1.2.3 Macrophage polarisation beyond the M1/M2 paradigm

Despite the widely accepted classification, it is now known that M1 and M2 are not the only macrophage subtypes (98). For example, polarisation of mouse bone marrow macrophages with LPS and ovalbumin immune complexes (IgG-OVA) generated a third type of macrophage population capable of secreting IL-10 but not IL-12 (99). This suggested that IgG-OVA polarised macrophages have a regulatory function. Based on this observation, it is hypothesized that at least three groups of macrophages exist: proinflammatory (M1), tissue-remodelling (M2), and immunoregulatory (99, 100). These last had been formerly included in the M2 category. Furthermore, polarisation with several other stimuli such as IL-10 and TGF-β followed by transcriptional profiling revealed even more subgroups (101). Thus, M1 and M2 are currently regarded as extremes of a broader functional continuum that spans various proinflammatory and tissue remodelling functions (100). As such, a more consistent macrophage nomenclature and a set of experimental guidelines were proposed in 2013 (102). This nomenclature expands the M1/M2 paradigm to include the outcome of a variety of cytokine induced polarisations.

Macrophage as opposed to T cell polarisation causes phenotypic changes as early as six hours after stimulation, which disappear at later time points (103). Furthermore, macrophage subsets are also highly plastic and their biology is heavily influenced by the tissue in which

they reside (86). Research on macrophage plasticity points to epigenetic mechanisms (104). For example, marks of active chromatin such as H3K4me3 selectively appear at the promoters of M2 genes shortly after stimulation with IL-4 or IL-13 (103). These are replaced by the repressive mark H3K27me3 once the stimulus disappears (103). These chromatin modifications are mediated by histone methylases like Jmjd3, whose expression is induced by IL-4 (103, 105). This suggests a functional explanation for macrophage plasticity, since the induction of different chromatin modifying enzymes by environmental cues could allow rapid shifts in macrophage function.

1.3 Cytokine induced polarisation mediates pathologic inflammation in autoimmunity

In addition to its fundamental role in the immune response against pathogens, cytokine induced cell polarisation is also involved in driving chronic inflammation (2). Evidence for the role of cytokines in autoimmune and autoinflammatory disease comes from different sources. For example, studies of experimental autoimmune encephalomyelitis (EAE), the animal model of multiple sclerosis (MS), have identified increased levels of IL-1β in cerebrospinal fluid of animals with active disease (106-108). This observation has also been recapitulated in cerebrospinal fluid of MS patients (108, 109). During the course of disease, IL-1β is produced by macrophages via the inflammasome pathway, among other myeloid cells (110, 111), and it has been suggested that it acts by polarising $CD4^+$ T cells to the Th17 phenotype (112). IL-1β also induces secretion of GM-CSF by Th17 cells (113, 114).

Moreover, targeting cytokines and their receptors with monoclonal antibodies is an efficient therapy for several diseases such as rheumatoid arthritis (RA) and psoriasis (2, 115). For example, blockade of IL-17 and IL-23 signalling is effective for treating psoriasis, which suggests the involvement of Th17 responses (116). Similarly, it has been demonstrated that patients with RA respond well to IL-6 blockade, which is also a cytokine involved in Th17 differentiation (117). More recently, subcutaneous administration of anti-IL17A monoclonal antibodies was proven efficient in the treatment of ankylosing spondylitis in a randomised trial with placebo controls (118).

Importantly, some cytokines associated to autoimmunity go beyond the classical definition of T cell and macrophage polarisation described above. For example, TNF blockade is to different extents a successful therapy for RA, giant-cell arteritis (GCA), Crohn's disease

(CD), ulcerative colitis (UC), psoriasis and ankylosing spondylitis (2, 119, 120). TNF is a cytokine of the innate immune response which induces secretion of inflammatory cytokines, apoptosis and necroptosis (119, 121, 122). Interestingly, TNF blockade in MS exacerbates disease (123). This is likely due to the regulatory functions of TNF in cells of the central nervous system (CNS) (124), although further research is necessary to investigate the role of TNF in MS. IL-21, IL-27, and type I IFN are also involved in RA, where they are thought to modify the function of $CD4^+$ T cells, especially follicular helper T (Tfh) cells (115, 125). In addition, it has been proposed that IL-23 might mediate autoimmune inflammation of the brain via its action in macrophages (126).

Despite the success of IL-6 and TNF blockade in RA, not all cytokines and receptors investigated have been efficient drug targets. For example, administration of subcutaneous IFN-β to patients with RA caused no changes in their radiological scores when compared to individuals treated with a placebo in a randomised, double blind trial (127). This suggests that IFN-β might not be crucial for RA pathology. Surprisingly, a randomised phase II study of intravenous and subcutaneous anti-IL17A antibody found no significant differences in ACR scores between patients with active RA who received the treatment and those who did not (128). This despite the same antibody being effective for treating ankylosing spondylitis (118). However, the treatment did achieve mild reduction of disease activity, C-reactive protein levels and self-reported pain (128), suggesting that IL-17 is involved in RA pathology but its blockade is not enough for treating the disease. Taken together, this evidence suggests that even though cytokine induced polarisation is central to autoimmunity, research is needed to design more precise and efficient therapeutic strategies. To achieve this, a deeper characterisation of the effect of cytokines across different cell types is necessary.

1.4 Project description

1.4.1 Project aims

It has become increasingly evident that a deeper cellular characterisation is necessary to explain how cytokines contribute to autoimmunity. This thesis describes a study that aimed to generate gene expression data of cytokine induced cell states in $CD4^+$ T cells and macrophages using RNA-sequencing (RNA-seq). Even though several studies have addressed this question before, they have either focused on mouse lymphocytes (129), assessed the cell states of interest without studying the process that generates them (130), been limited to very few states (131), or sampled subsets of the transcriptome (94). We

believe that this study contributes to fill this gap by asking fundamental questions of cytokine biology not previously answered for human immune cells. Specifically, we asked if previously described subsets of cells such as Th1, Th2, Th17 or M1 and M2 macrophages showed a stable or a transitional transcriptome, and how their gene expression changed throughout time. We wanted to determine if any evident gene expression program existed and at which time point it was triggered. We also aimed to characterise the key aspects of these cell states, extending the definition of cell subsets, currently relying almost solely on cytokine secretion profiles. Furthermore, we wanted to determine which cell type was affected by cytokines associated with autoimmunity, as well as which genes or pathways were activated by these cytokines. This has, to our best knowledge, not been assessed in these cell types before. By doing this, we wanted to determine if any of these cytokines could be a suitable drug target for the autoimmune diseases under consideration.

1.4.2 Experimental design

In order to achieve our aims, we devised an experimental design based on low coverage RNA-seq. Briefly, we stimulated and polarised naive $CD4^+$ T cells and monocyte derived macrophages from two healthy individuals using cytokines associated to autoimmunity. Details of the stimulatory conditions are presented in the methodology section. Macrophages were analysed after six hours, while T cells were assessed 16 hours and five days after stimulation. Next, we isolated RNA and performed low coverage RNA-seq, and used these data to carry out a differential gene expression analysis between cells stimulated in the presence and in the absence of cytokines. Finally, we identified pathways, TFs, and gene co-expression modules relevant to these cell states (**Figure 1.1**).

It has been shown that the statistical power of RNA-seq studies increases in proportion to gene coverage and sample size (132-134), being this last factor the most important determinant of sensitivity (133). In order to account for the caveats in our experimental design, which has both low sample size and low coverage, we used the statistical method for differential expression analysis “DESeq2”, specifically designed for low sample size (135) and which has been shown to achieve good power under similar circumstances (132). A thorough analysis of the statistical power of this study was also carried out and is presented in the results section.

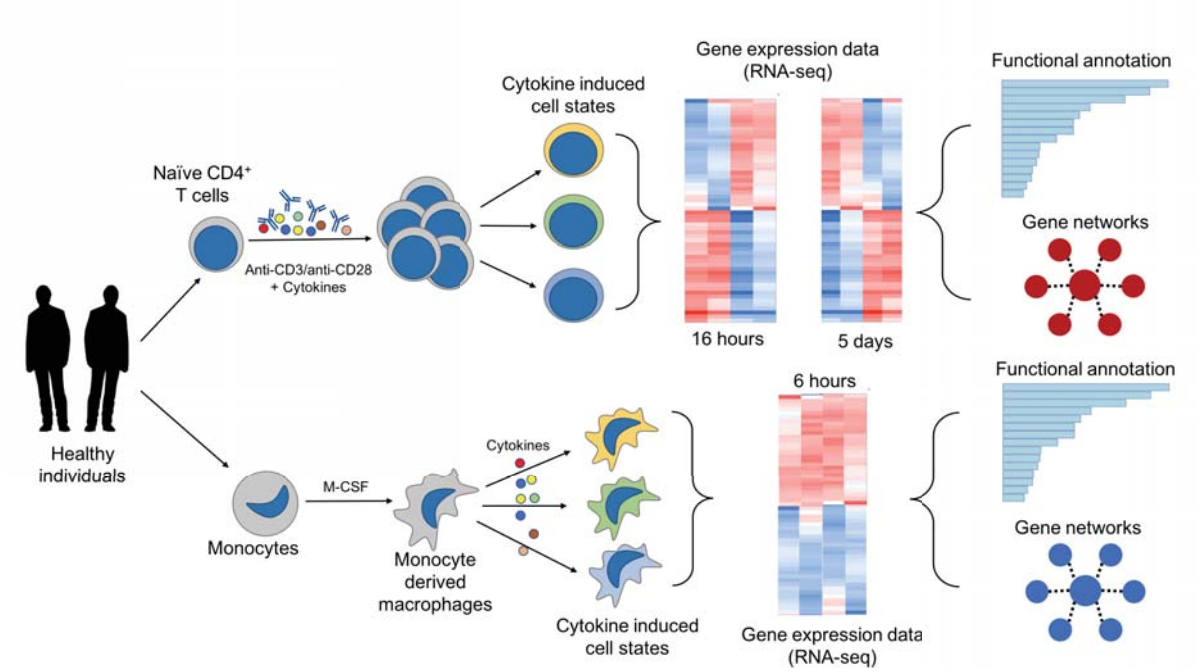

Figure 1.1 Overview of the experimental design In this study, two cell types were isolated from two healthy blood donors. Cytokine induced polarisation followed by RNA sequencing was performed on these cells. The resulting data was used to perform differential expression analysis, pathway enrichment analysis, and gene co-expression network analysis.

1.5 Thesis outline

In chapter 2, I present an in-depth description of the experimental methodology and data analysis techniques. Chapter 3 contains the results from optimisation experiments used to determine the optimal conditions for isolation, cell culture, and stimulation of human immune cells. Subsequently, chapter 4 describes the results from RNA-seq data analysis, including statistical power estimations, differential gene expression analysis and identification of relevant pathways and TFs. Finally, chapter 5 summarises the conclusions of the study and presents a brief discussion on future perspectives.